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Growth of Macrophage-Tropic and Primary Human Immunodeficiency Virus Type 1 (HIV-1) Isolates in a Unique CD4$^+$ T-Cell Clone (PM1): Failure To Downregulate CD4 and To Interfere with Cell-Line-Tropic HIV-1

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Human immunodeficiency virus type 1 (HIV-1) isolates derived directly from clinical samples are usually unable to grow in cytokine-independent continuous cell lines, thus hindering the study of their biological features and their sensitivity to humoral and cellular protective immunity. To overcome these limitations, we have derived from the Hut78 T-cell line a CD4$^+$ clone (PM1) characterized by a unique susceptibility to a wide range of HIV-1 isolates, including primary and biologically pure macrophage (Mϕ)-tropic isolates (e.g., HIV-1_{BaL}), which are unable to infect other human T- or promonocytic cell lines. Both primary and Mϕ-tropic HIV-1 establish persistent infection in PM1, with sustained levels of virus replication for prolonged periods. Experiments with chimeric viruses containing envelope fragments of HIV-1_{BaL} inserted into the genetic framework of HXB2, a molecular clone derived from the cell-line-tropic isolate HIV-1_{IIIB}, showed the third hypervariable domain (V3) of gp120 to be a critical determinant of the cell line tropism of HIV-1. Nevertheless, the V3 loop of HIV-1_{BaL} was not sufficient to confer on the chimeras a bona fide Mϕ tropism. The biological characteristics of HIV-1_{BaL} and of a primary isolate (HIV-1_{573}) were investigated by using the PM1 clone. Infection of PM1 by HIV-1_{BaL} was critically dependent on the CD4 receptor, as shown by competition experiments with an anti-CD4 monoclonal antibody (OKT4a) or with soluble CD4. However, the amount of soluble CD4 required for inhibition of HIV-1_{BaL} was approximately 100-fold higher than for HIV-1_{IIIB}, suggesting that the affinity of HIV-1_{BaL} for CD4 is significantly lower. Infection of PM1 with either HIV-1_{BaL} or HIV-1_{573} failed to induce downregulation of surface CD4 expression and syncytium formation. Analogous results were obtained with a chimeric virus (HXB2[BaL PvuII-BamHI]) encompassing a large portion of gp120 and gp41 of HIV-1_{BaL}, indicating that the *env* genes contain critical determinants for CD4 downregulation and syncytium formation. Consistent with the lack of CD4 downregulation, persistent infection of PM1 by HIV-1_{BaL} or HIV-1_{573} failed to interfere with HIV-1_{IIIB} superinfection, as revealed by the expression of a type-specific V3 loop epitope (M77) and by the induction of extensive syncytium formation. This lack of interference suggests that a direct viral interaction may occur in vivo between biologically diverse HIV-1 strains. The PM1 clone represents a reproducible and efficient cellular system for the in vitro propagation of primary and Mϕ-tropic isolates of HIV-1 and may therefore provide a precious tool for studies aimed at developing broadly active vaccines against HIV-1.

Similar to other RNA viruses, human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, is characterized by a high degree of genetic variability. This heterogeneity is particularly marked in some regions of the viral genome, such as the major envelope gene, gp120, and exists not only among isolates derived from different patients (1, 15, 40, 47) but also, albeit more restricted, among clones derived from the same individual (13, 16, 27, 33, 39). In this latter case, the viral population, which is constantly modulated under the selective pressure of the host's immune system, has been referred to as a quasispecies (13). The diverse viral isolates have been categorized on the basis of their in vitro biological properties, i.e., cellular tropism (T cells versus mononuclear phagocytes [Mϕ]), rate or extent of replication (high versus low), and ability to induce polykarion formation (syncytium inducing [SI] versus non-syncytium inducing [NSI]) (2, 4, 7, 36). Although the genetic basis for this heterogeneity has yet to be definitively elucidated, some genetic determinants, such as the third hypervariable domain (V3) of gp120, have been consistently associated with specific biological features, including the ability to grow in Mϕ or in continuous cell lines (10, 19, 46). Whether, and to what extent, the variation of HIV-1 in vivo can influence the progression of the disease is still a matter of debate. The main clinical observation supporting this concept is the fact that highly cytopathic viral isolates (SI) are recovered more frequently from patients in the advanced stages of the disease (2, 4, 7, 34, 42).

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The majority of the HIV isolates obtained directly from patients' specimens, particularly during the early stages of the disease, are unable to replicate in continuous cell lines, whereas they can be propagated, with variable efficiency, in activated peripheral blood mononuclear cells (PBMC) and/or in primary Mϕ cultures. Isolates that replicate efficiently in Mϕ are usually characterized by a less pronounced cytopathic effect in vitro and an inability to grow in cell lines (2, 12, 35, 36, 48). To date, comparative studies of biologically diverse isolates have been hampered by the lack of uniform and well-standardized cellular systems for their in vitro propagation. The only cellular system which is consistently susceptible to both primary and laboratory-passaged HIV isolates with either Mϕ or T-cell tropism, i.e., activated PBMC, is a mixed cellular population containing both T cells and Mϕ. In this study, we describe a continuous $CD4^+$ T-cell clone (PM1) with a unique susceptibility to both Mϕ-tropic and primary HIV-1 isolates. Taking advantage of this cellular system, we have determined that Mϕ-tropic, NSI isolates, most likely because of a lower affinity for the CD4 receptor, fail to downregulate CD4, to induce polykarion formation, and to interfere with superinfection by cell-line-tropic HIV-1. In addition, using chimeric viruses, we have mapped to the V3 region of gp120 the ability of HIV-1 to grow in continuous cell lines, although this region was insufficient to confer on the chimeras a bona fide Mϕ tropism. The PM1 cell line may represent a useful tool for studies related to the generation of broadly reactive vaccines effective against primary and Mϕ-tropic HIV isolates.

MATERIALS AND METHODS

Cells. Clone PM1 was obtained in our laboratory, using the limiting dilution cloning technique, from the neoplastic T-cell line Hut78 (American Type Culture Collection, Rockville, Md.). It shows phenotypic characteristics similar to those of the parental cell line (i.e., $CD3^+$ $CD4^+$ $CD8^-$ $CD26^+$ $HLA\text{-}DR^+$), but it exhibits a higher CD45RO and a lower CD45RA surface membrane expression. The other $CD4^+$ T-cell lines and the promonocytic cell line U937 were obtained from the American Type Culture Collection. PBMC and adherent Mϕ were prepared from the peripheral blood of healthy adult volunteers as follows. The mononuclear cell fraction was recovered after centrifugation of concentrated leukocyte preparations, previously diluted 1:3 in sterile phosphate-buffered saline (PBS), over a Ficoll (Pharmacia, Piscataway, N.J.) gradient. The cells recovered from the gradient were extensively washed by low-speed centrifugation in a large volume of PBS to remove platelets and eventually resuspended in RPMI medium with 10% (vol/vol) fetal bovine serum. PBMC were resuspended at 2×10^6 cells/ml and stimulated with purified phytohemagglutinin (Wellcome, Beckenham, United Kingdom) at 1 μg/ml. Partially purified human interleukin 2 (Boehringer Mannheim, Indianapolis, Ind.) was added at 10 U/ml after 48 h.

For the establishment of adherent Mϕ, the mononuclear cells were seeded in 75-mm^3 flasks (Costar, Cambridge, Mass.) at a concentration of 5×10^6/ml in the presence of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (Boehringer Mannheim) at 10 U/ml. Three days later, one half of the culture volume, including one half of the nonadherent cells, was removed and replaced by fresh complete medium containing GM-CSF. After 4 to 6 additional days, depending on the confluence of the adherent cell population, all the nonadherent cells were carefully removed and the adherent monolayer was washed several times by vigorous pipetting with prewarmed PBS containing 10% fetal bovine serum. After pelleting the nonadherent cells, the original conditioned medium, diluted 1:4 in fresh medium containing GM-CSF, was added back to the adherent cell cultures.

Viruses and infection procedures. HIV-1_{BaL} (12) was serially passaged in primary adherent Mϕ cultures totally devoid of detectable contaminating T cells. HIV-1_{IIIB}, one of the strains now collectively referred to as HIV-1_{LAI}, was derived from the culture supernatant of chronically infected H9 cells. The primary HIV-1 isolates were obtained after cocultivation of phytohemagglutinin-activated patients' PBMC with phytohemagglutinin-activated PBMC from healthy blood donors at a 1:1 ratio. For infection, the viral stocks were normalized to approximately 1 ng of p24 core antigen per ml, as assessed by a commercial enzyme-linked immunosorbent assay (ELISA) (DuPont, Boston, Mass.), and 1 ml was used to infect 10^6 cells. After 1 h at 37°C, the cells were washed repeatedly and then recultured in complete medium. The medium was supplemented with 10 U of interleukin 2 per ml for PBMC or with 10 U of GM-CSF per ml for Mϕ. The level of virus replication was assayed by measuring the amount of extracellular p24 antigen by ELISA.

Two chimeric viruses, HXB2[BaL PvuII-BamHI] and HXB2[BaL V3], were generated. For the construction of the first chimera, we amplified by PCR, from a partial molecular clone of HIV-1_{BaL}, a region corresponding to the *Pvu*II-*Bam*HI fragment of the envelope gene of clone pHXB2, derived from HIV-1_{IIIB} (6, 8). The primers were designed to introduce the appropriate restriction enzyme sites into the amplified fragment, in order to allow its insertion into the *Pvu*II-*Bam*HI site of a *Sal*I-*Bam*HI subclone of pHXB2. The *Sal*I-*Bam*HI chimeric fragment was then ligated into the large *Sal*I-*Bam*HI fragment of pHXB2. To construct the HXB2[BaL V3] chimera, we utilized a V3 cassette of the *Sal*I-*Bam*HI subclone of pHXB2 from which the V3 coding region had been removed and into which unique *Mlu*I and *Hpa*I restriction sites were inserted (25). Defined V3 loop regions were synthesized as a set of four overlapping oligonucleotides which were then annealed and ligated into the *Mlu*I and *Hpa*I sites of the cassette. The cassette was inserted into the *Sal*I-*Bam*HI subclone to make a complete infectious clone containing the heterologous V3 loop. The amino acid sequence of the V3 loop of HIV-1_{BaL} was CTRPNNNTRKSIHIG PGRAFYTTGEIIGDIRQAHC. The correct insertion of the fragments was verified by DNA sequencing. It is noteworthy that the sequence of HIV-1_{BaL} obtained in our laboratory had a single amino acid change (an L→F substitution at the tip of the loop) with respect to that published by Hwang and colleagues, which displayed a different degree of sensitivity to soluble CD4 (sCD4) neutralization (20).

Fluorocytometry, radioimmunoprecipitation, and electron microscopy. The monoclonal antibodies (MAbs) used in this study were phycoerythrin-conjugated Leu3a (anti-CD4) (Becton Dickinson, Mountain View, Calif.), OKT4a and phycoerythrin-conjugated OKT4 (anti-CD4) (Ortho Diagnostics, Raritan, N.Y.), M26 (anti-HIV-1 p24), M77 (anti-HIV-1_{IIIB} gp120 V3 loop; type specific), and M90 (anti-HIV-1 gp120; group specific). Fluorescein isothiocyanate-conjugated goat antiserum to murine immunoglobulin G (Sigma, St. Louis, Mo.) was used as a second-layer antibody. Intracellular staining was performed after fixation of the cells with 2% paraformaldehyde for 10 min at room temperature followed by 70% ethanol at −20°C for 30 min. Fluorocytometric analysis was performed on a FACScan analyzer (Becton Dickinson). Control tests were stained with a mouse immunoglobulin G subclass 1 irrelevant MAb conjugated with either fluorescein or phycoerythrin (Becton Dickinson). At least 5,000 events were accumulated in each test. For radioimmunoprecipitation, infected and uninfected cells (10^6/ml) were radiolabeled by incubating at 37°C for 7 h in methionine-free medium containing [^{35}S]methionine (100 μCi/ml). Labeled cells were washed with PBS and disrupted at 4°C in PBS containing 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and 0.5% sodium deoxycholate (PBS-TDS). The lysate was adsorbed for 4 h at 4°C with 0.2 ml of 10% protein A Sepharose and 10 μl of normal human serum and was clarified by centrifugation. Immunoprecipitation reactions were performed by the addition of 10 μl of anti-HIV-1 antibody-positive human serum and 0.2 ml of 10% protein A Sepharose to 1 ml of the clarified cell extract. The samples were incubated for 18 h at 4°C, and the immunoprecipitates were collected by centrifugation at 2,000 × *g* for 10 min. The pellet was repeatedly washed in PBS-TDS, resuspended in 75 μl of 0.065 M Tris-HCl (pH 6.7)–1% SDS–10% glycerol–2.5% β-mercaptoethanol–0.1% bromophenol blue, heated for 3 min at 90°C, and analyzed by SDS-polyacrylamide gel electrophoresis. For electron microscopy analysis, the cells were repeatedly washed with PBS, pelleted, and fixed with a solution containing 0.1 M sodium cacodylate and 1.25% glutaraldehyde. Transmission electron microscopy was performed according to standard procedures.

Syncytium assay. The syncytium assay was performed by cocultivating equal numbers (10^5/well) of infected and uninfected cells in 24-well plates (Costar) in a total volume of 1 ml of complete medium. Either PM1 or SupT1 cells were used as uninfected partners in separate experiments. Syncytium formation was scored visually after 12, 24, and 48 h.

Infection inhibition with anti-CD4 antibodies or sCD4. MAb OKT4a, previously dialyzed to remove sodium azide and sterilized by filtration through a 0.2-μm-pore-size membrane, was used at 5 μg/ml to pretreat 10^6 test cells for 30 min at 4°C. The cells were then incubated for 1 h at 37°C with the virus stock (1 ng/ml). sCD4 (DuPont), previously sterilized by filtration, was used at various concentrations to pretreat the virus stocks for 30 min at 37°C. The pretreated virus was then used to infect 10^6 cells. After 1 h at 37°C, the cells were washed three times with cold RPMI and resuspended in complete culture medium containing OKT4a or sCD4 at the same original concentration.

RESULTS

Susceptibility of PM1 to selectively Mϕ-tropic and primary HIV-1 isolates. In an attempt to identify a continuous cell line susceptible to Mϕ-tropic, NSI HIV-1 isolates, we cloned the Hut78 cell line by the limiting dilution cloning technique and screened the clones on the basis of their infectibility by a selectively Mϕ-tropic isolate, HIV-1_{BaL}. Only one of several hundreds of clones tested was found to be highly susceptible to productive infection by HIV-1_{BaL}. The clone was designated PM1. Beginning 3 days after exposure to the viral stock, increasing levels of both HIV-1 p24 antigen and reverse tran-

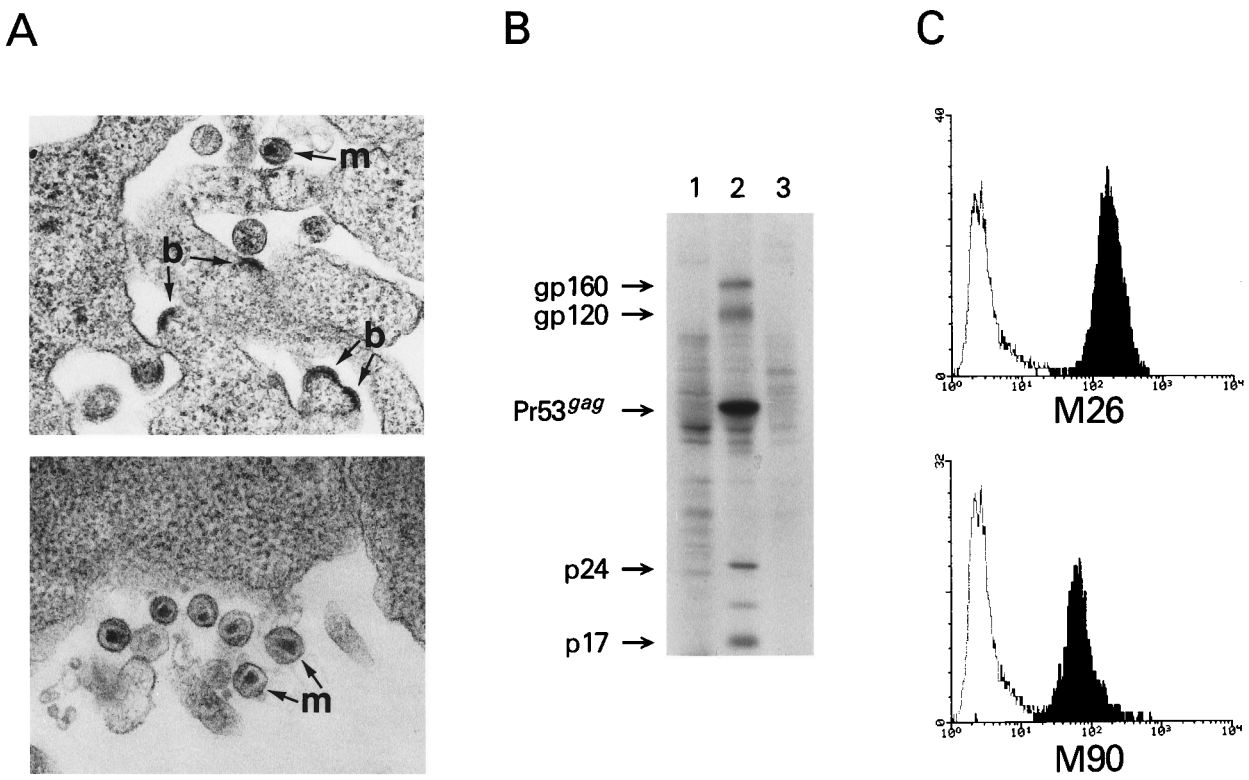

FIG. 1. (A) Electron micrographs illustrating HIV-1_{BaL} virions released by chronically infected PM1 cells. Both budding (b) and mature (m) viral particles can be seen. (B) Radioimmunoprecipitation assay of $PM1_{BaL}$ metabolically labeled with [^{35}S]methionine. Lane 1, human anti-HIV antibody-negative control serum; lane 2, human anti-HIV antibody-positive serum; lane 3, human anti-HIV antibody-positive serum on uninfected PM1, used as a control. (C) Fluorocytometric analysis of intracellular HIV-1 p24 (M26) and surface membrane gp120 (M90) expression in $PM1_{BaL}$.

scriptase activity were observed in the culture supernatants (not shown). Electron microscopic analysis of infected cells (Fig. 1A) demonstrated the presence of abundant budding viral particles on the cell surface, as well as of mature virions in the extracellular space. The infection was confirmed by radioimmunoprecipitation with an antibody-positive human serum (Fig. 1B), by immunofluorescence staining with an anti-HIV-1 p24 MAb (M26; cytoplasmic immunofluorescence) and an anti-HIV-1 gp120 MAb (M90; surface membrane immunofluorescence) (Fig. 1C), and by the detection of specific HIV-1 genetic sequences by PCR (not shown).

To test the susceptibility of the PM1 clone to different HIV-1 isolates, we used a panel of primary isolates, previously passaged in vitro only in phytohemagglutinin-activated PBMC. Several other T-cell lines (i.e., CEM, Jurkat, Hut78, SupT1, H9, Molt-3, Molt-4) and a promonocytic cell line (U937) were tested in parallel. All the cell lines were exposed to identical viral inocula and monitored for up to 4 weeks after infection by testing the amount of extracellular p24 release. All the HIV isolates included in this study were able to grow efficiently in primary PBMC, whereas only three of eight isolates replicated well in Mϕ cultures. As summarized in Table 1, none of the cell lines, with the exception of CEM for one isolate (no. 3), was susceptible to infection by any of the primary HIV-1 isolates tested. In contrast, PM1 was rapidly and efficiently infected by five of the primary isolates (no. 1 through 4 and 8). No correlation was observed between the ability to grow in primary Mϕ and the ability to grow in PM1. Moreover, PM1 was readily infected by several HIV-2 and simian immunodeficiency virus isolates tested (not shown).

Critical role of the V3 domain of gp120 in the cell line tropism of HIV-1. To elucidate whether the determinants responsible for the growth of HIV-1 in continuous cell lines are genetically specified and map to selected regions of the viral envelope, two chimeric recombinant viruses were constructed. The first, HXB2[BaL PvuII-BamHI], contains a 1.3-kb *Pvu*II-*Bam*HI fragment of the envelope of HIV-1_{BaL} inserted into the pHXB2 infectious molecular clone, originally derived from isolate HIV-1_{IIIB} (6, 8). The 1.3-kb fragment includes the third hypervariable domain (V3) of gp120, the suggested CD4-binding domain, part of a region previously indicated as critical for HIV-1 replication in human Mϕ (19, 23, 30, 38, 45, 48), and a large 5′ portion of the gp41 gene, including the sequences coding for the fusogenic domain (11, 18). The second chimera, HXB2[BaL V3], contains the V3 domain of gp120 of HIV-1_{BaL} inserted into the pHXB2 clone of HIV-1_{IIIB}. A schematic diagram of the two chimeric viruses is presented in Fig. 2.

The HXB2[BaL PvuII-BamHI] and HXB2[BaL V3] chimeras were biologically active, as demonstrated by their ability to grow efficiently in human PBMC. As shown in Table 1, both chimeric viruses exhibited exactly the same cell line tropism as the original HIV-1_{BaL} isolate. They grew in the PM1 cell line with the same efficiency as HIV-1_{BaL} but failed to replicate in any of the other $CD4^+$ T-cell or promonocytic cell lines tested. Thus, genetic determinants in the viral envelope and, in particular, in the V3 domain of gp120 control the ability of HIV-1 to infect different continuous human cell lines. In contrast to HIV-1_{BaL}, however, neither of the two chimeras were able to efficiently replicate in primary Mϕ cultures, indicating that the

TABLE 1. Cellular host range of the HIV-1 strains used in this study

HIV-1 isolate	Virus replication[a] in different cell types[b]										
	PBMC	Mϕ	CEM	JR	H78	ST1	H9	M3	M4	U937	PM1
HIV-1_{BaL}	++	++	—	—	—	—	—	—	NT[c]	—	++
HIV-1_{IIIB}	++	—	++	++	++	++	++	++	++	++	++
Primary isolates (no.)											
1	++	—	—	NT	NT	—	—	NT	NT	—	++
2	++	—	—	NT	NT	—	—	NT	NT	—	++
3	++	—	++	NT	NT	—	—	NT	NT	—	++
4	++	++	—	—	—	—	—	—	—	NT	++
5	++	+	—	—	—	—	—	—	—	NT	—
6	++	+	—	—	—	—	—	—	—	NT	—
7	++	—	—	—	—	—	—	—	—	NT	—
8	++	—	—	—	—	—	—	—	—	NT	++
Chimeric viruses											
HBX2[BaL PvuII-BamHI]	++	±	—	—	—	—	—	—	NT	—	++
HBX2[BaL V3]	++	±	—	NT	NT	—	—	NT	NT	—	++

[a] ++, >10 ng of extracellular p24 per ml for at least 2 consecutive weeks; +, 1 to 10 ng/ml; ±, <1 ng/ml; —, no viral replication.
[b] JR, Jurkat; H78, Hut78; ST1, SupT1; M3, Molt-3; M4, Molt-4.
[c] NT, not tested.

V3 domain of gp120 is not by itself sufficient to confer a bona fide Mϕ tropism.

CD4 dependence of infection of PM1 by HIV-1: different susceptibility of Mϕ-tropic and cell-line-tropic isolates to sCD4 neutralization. To ascertain whether infection of PM1 by biologically diverse HIV-1 isolates was dependent upon the CD4 receptor, competition experiments were performed with MAbs OKT4a and sCD4. As shown in Table 2, acute infection by either HIV-1_{IIIB} or HIV-1_{BaL} was completely inhibited by OKT4a used at 5 μg/ml, indicating that CD4 is critical for infection of PM1 by both cell-line- and Mϕ-tropic HIV-1 isolates. To further investigate the role of CD4 in the infection of PM1 by HIV-1_{BaL} or HIV-1_{IIIB}, as well as to evaluate the affinity of divergent HIV-1 isolates for the CD4 receptor, competition experiments were performed by pretreating the virus inoculum with recombinant sCD4. As indicated in Table 2, sCD4 totally blocked infection of PM1 by both viral strains. However, the amount of sCD4 required to inhibit by more than 90% the level of infection by HIV-1_{BaL} was approximately 100-fold higher than that required to likewise inhibit HIV-1_{IIIB} infection. This finding suggests that the affinity of the HIV-1_{BaL} virions for CD4 is lower than that of HIV-1_{IIIB}. These results are consistent with those of Hwang and colleagues (20), although their study reported only a 10-fold difference in susceptibility to sCD4 neutralization between HIV-1_{BaL} and HIV-1_{IIIB}. However, it is noteworthy that the V3 loop sequences obtained in the two laboratories differ by one amino acid (an L→F substitution at the tip of the loop). Indeed, the insertion of a V3 loop bearing the F substitution into an HIV-1_{IIIB}-derived molecular clone yielded an sCD4 neutralization susceptibility comparable to that observed with our HIV-1_{BaL} isolate (20).

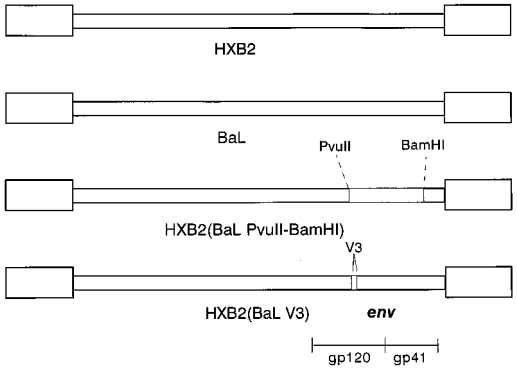

FIG. 2. Schematic representation of the chimeric recombinant viruses HXB2[BaL PvuII-BamHI] and HXB2[BaL V3] containing fragments of the envelope of $PM1_{BaL}$ inserted into the pHXB2 framework.

Persistent infection of PM1 by Mϕ-tropic, NSI HIV-1 isolates: long-term virus production and lack of syncytium formation. Approximately 2 weeks after exposure of PM1 to HIV-1_{BaL} or to a primary isolate, HIV-1_{573} (Table 1, isolate no. 1), more than 99% of the cells displayed signs of productive infection, as conclusively shown by intracellular staining with the anti-HIV core MAb M26 (Fig. 1C). In spite of the high levels of viral replication, however, infected PM1 cultures failed to exhibit signs of giant multinucleated cell formation at any time after infection with these viruses. Only a very limited single-cell cytopathic effect was observed (most likely due to programmed cell death mechanisms), which peaked at day 6 to 8 postinfection (5 to 10% of the cells) and persisted for an additional week (data not shown). Subsequently, the cell number continued to grow with kinetics similar to those of the uninfected PM1 counterpart. The proportion of virus-expressing cells remained constant during long-term follow-up of infected cul-

TABLE 2. Inhibition of HIV-1_{BaL} and HIV-1_{IIIB} infection in PM1 cells by OKT4a and sCD4

Strain	HIV-1 p24 antigen (pg/ml) released after treatment with[a]:					
	None (control)	sCD4 (μg/ml)				OKT4a (5 μg/ml)
		0.4	4	40	400	
HIV-1_{BaL}	>500	>500	>500	255	<5	<5
HIV-1_{IIIB}	>500	225	<5	<5	<5	<5

[a] Antigen released into the culture supernatants at day 8 postinfection.

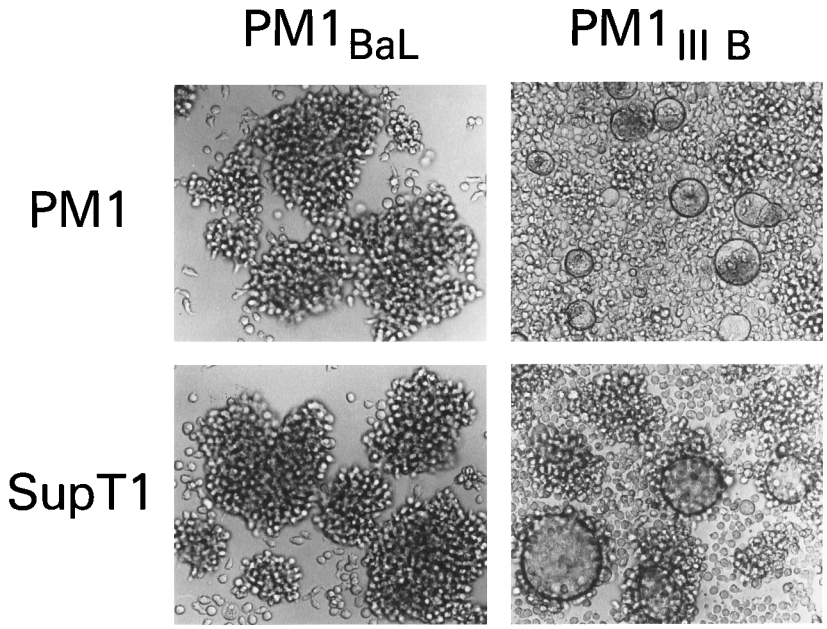

FIG. 3. Syncytium assay of $PM1_{BaL}$ or $PM1_{IIIB}$. The cells were cocultured at a 1:1 ratio for 24 h with the uninfected $CD4^+$ human T-cell lines PM1 or SupT1.

tures (up to 120 days postinfection), and the cells continued to produce high levels of infectious extracellular virus. Similar observations were made after infection of PM1 with the HXB2[BaL PvuII-BamHI] chimera. In contrast, infection with a typical cell-line-tropic isolate ($HIV\text{-}1_{IIIB}$) rapidly induced in PM1 profound cytomorphological changes (i.e., syncytium formation) and massive cellular death. Similarly, syncytium formation was observed after infection with the HXB2[BaL V3] chimera, indicating that the V3 loop alone is not sufficient to confer an NSI phenotype.

Consistent with the lack of syncytium formation observed during acute infection, PM1 persistently infected by $HIV\text{-}1_{BaL}$ ($PM1_{BaL}$), $HIV\text{-}1_{573}$ ($PM1_{573}$) or the HXB2[BaL PvuII-BamHI] chimera ($PM1_{HXB2[BaL\ PvuII\text{-}BamHI]}$) failed to induce the formation of giant multinucleated cells in a conventional syncytium assay performed by cocultivating the cells for up to 48 h with uninfected PM1 or SupT1 (Fig. 3). In contrast, PM1 persistently infected by $HIV\text{-}1_{IIIB}$ ($PM1_{IIIB}$) or HXB2[BaL V3] were potent syncytium inducers.

Lack of CD4 downregulation by Mϕ-tropic, NSI HIV-1 isolates. The surface membrane expression of CD4 and HIV-1 gp120 was analyzed in $PM1_{BaL}$ and $PM1_{IIIB}$ by two-color fluorocytometry. As shown in Fig. 4, uninfected PM1 exhibited CD4 (Leu3a) on the vast majority (>90%) of the cells. As expected, CD4 was completely downregulated in $PM1_{IIIB}$, which expressed gp120 (M90) on the surface membrane of more than 90% of the cells. In contrast, virtually no downregulation of CD4 expression was observed with $HIV\text{-}1_{BaL}$, despite the high level of expression of surface HIV-1 gp120 (>90% of the cells). The results were confirmed by using two additional anti-CD4 MAbs, OKT4a and OKT4, that recognize different immunoglobulin-like domains of CD4 (V1 and V3/4, respectively) (data not shown). Similar results were obtained with the $HIV\text{-}1_{573}$ primary isolate and with the HXB2[BaL PvuII-BamHI] chimera (data not shown). This result demonstrates that the envelope genes of HIV-1 contain critical determinants for the induction of CD4 downregulation.

Lack of interference between Mϕ-tropic, NSI and cell-line-tropic HIV-1 isolates. Because infection by Mϕ-tropic, NSI HIV-1 failed to downregulate the expression of CD4, we investigated whether interference occurred between these viruses and $HIV\text{-}1_{IIIB}$. $PM1_{BaL}$ (exhibiting HIV-1 p24 in more

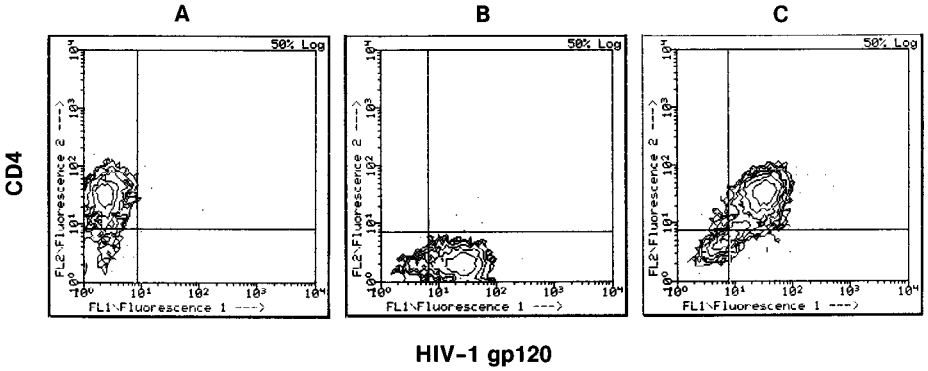

FIG. 4. Two-color fluorocytometric analysis of membrane CD4 (Leu3a) and gp120 (M90) expression in uninfected PM1 (A), $PM1_{IIIB}$ (B), and $PM1_{BaL}$ (C) cells.

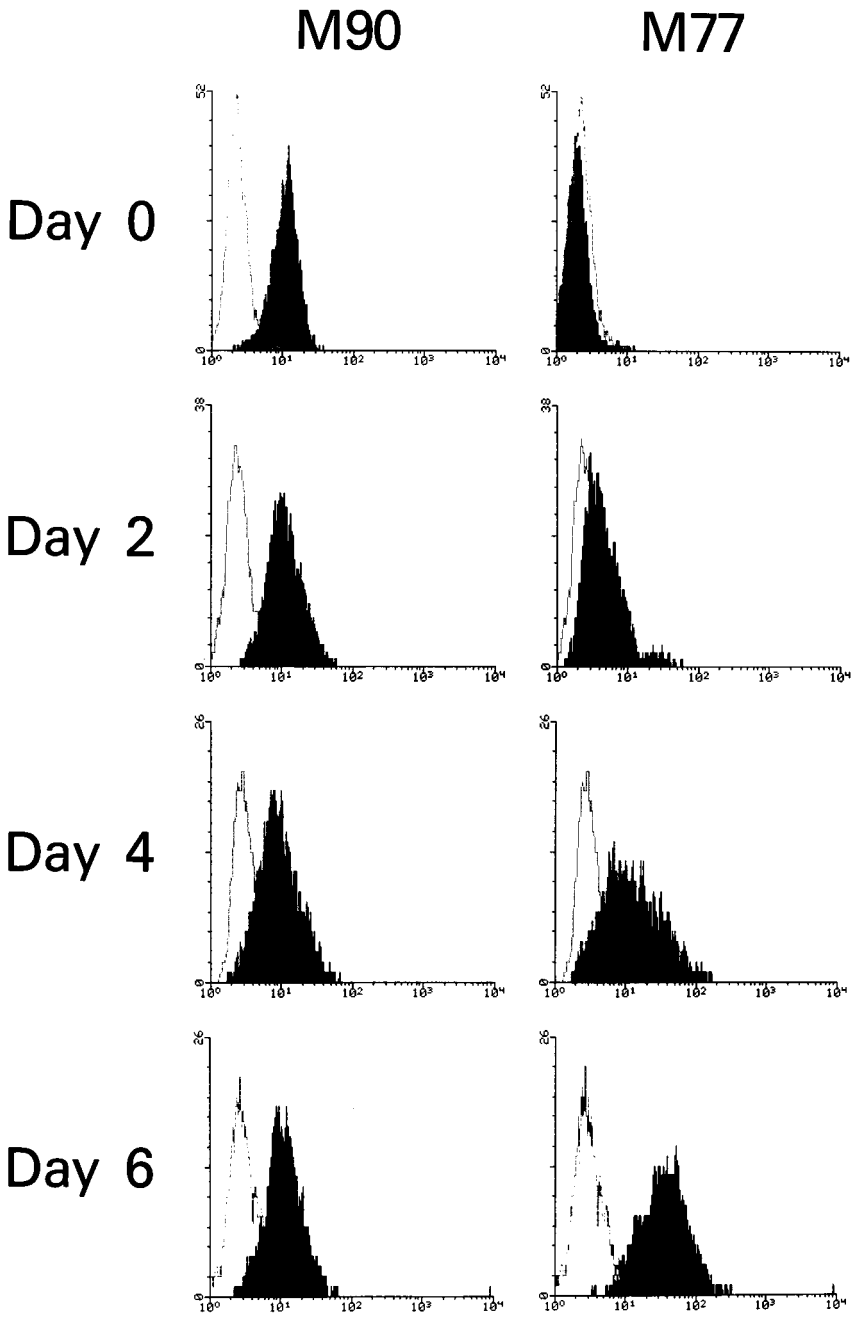

FIG. 5. Lack of receptor interference between HIV-1_{BaL} and HIV-1_{IIIB}. Fluorocytometric analysis of surface HIV-1 gp120 expression with MAbs M90 (group specific; reactive with both HIV-1_{BaL} and HIV-1_{IIIB}) and M77 (type specific; reactive only with HIV-1_{IIIB}) at various days after superinfection of $PM1_{BaL}$ with HIV-1_{IIIB}.

than 99% of the cells, by cytoplasmic fluorocytometry) was exposed to HIV-1_{IIIB} at 1 ng of p24 per 10^6 cells. Productive superinfection by HIV-1_{IIIB} was rapidly established, as clearly indicated in a time course fluorocytometric analysis by staining with MAb M77, a type-specific MAb directed to HIV-1_{IIIB} gp120 which fails to recognize any of the other HIV-1 strains tested (Fig. 5). Superinfection of $PM1_{BaL}$ by HIV-1_{IIIB} was accompanied by the induction of a dramatic cytopathic effect with extensive syncytium formation and massive cellular death. A similar lack of interference was observed when HIV-1_{IIIB} was used to superinfect $PM1_{573}$ or $PM1_{HXB2[BaL\ PvuII\text{-}BamHI]}$ (data not shown). These data demonstrate that Mϕ-tropic, NSI isolates do not interfere with cell-line-tropic, SI HIV-1.

DISCUSSION

The pathogenic mechanism of AIDS, leading to the progressive and irreversible depletion of $CD4^+$ T cells and, eventually, to the generalized collapse of the immune system, is still poorly understood. One of the major unsolved questions is the role played in the disease progression by the high degree of genotypic and phenotypic heterogeneity observed among different

HIV-1 isolates, even in individual patients (1, 2, 7, 9, 13, 15, 16, 27, 33, 39, 40, 47). This heterogeneity may result in the continuous emergence of neutralization escape mutants and/or of viral variants with increased pathogenicity during the course of the infection. This latter concept is corroborated by the more frequent recovery of highly cytopathic (SI) HIV strains from patients in the advanced stages of the disease (2, 4, 7, 34, 42). In contrast, NSI viral strains, characterized by a lesser cytopathogenic potential and, frequently, by a tropism for Mϕ, are most commonly detected during the early phases of the infection, even though they usually persist in patients with late-stage disease (2, 7, 35). In fact, it has been shown that early seroconverters harbor almost exclusively Mϕ-tropic, NSI isolates, which are usually characterized by a high degree of genetic homogeneity in the V3 loop of gp120 (49). However, it is also important to underline that a tissue-specific distribution of HIV-1 strains with different biological and genotypic characteristics has been demonstrated, with Mϕ-tropic, NSI isolates representing the predominant viral population in selected tissues, such as the central nervous system (21, 23, 37, 44).

The extreme variability of HIV-1 certainly represents a fundamental obstacle for the development of effective anti-HIV vaccines. A major issue, in this respect, is the different susceptibilities to antibody-mediated neutralization of laboratory-adapted viral strains and primary isolates recovered directly from patients' specimens (3). To date, the study of primary HIV-1 isolates, as well as of selectively Mϕ-tropic isolates, has been hampered by the lack of well-standardized cellular systems susceptible to a wide-range of viral strains. Indeed, the only cells that can be infected by biologically diverse HIV-1 isolates are activated primary PBMC, a cellular system that is heterogeneous (containing both T cells and Mϕ), costly, sometimes inefficient, and not consistently reproducible. We have described here a $CD4^+$ T-cell clone (PM1) that is remarkably susceptible to infection by a wide variety of HIV isolates, including primary and selectively Mϕ-tropic strains. The biological and molecular basis for this peculiar susceptibility of PM1, compared with that of the parental cell line (Hut78) or other sibling clones (e.g., H9), is at present unknown. The PM1 cellular system provides the unique opportunity of growing biologically diverse HIV isolates at high levels and for prolonged periods of time in a single in vitro-immortalized cell type. For these reasons, PM1 could be an extremely useful target cell for studies related to the development of broadly active anti-HIV vaccines.

The efficient growth of Mϕ-tropic, NSI isolates in PM1 cells permitted us to investigate their biological properties in a controlled and reproducible model system. Infection with such isolates induced neither the formation of giant multinucleated cells nor downregulation of CD4. It must be underlined that our HIV-1_{BaL} stock exhibits biologically pure characteristics, most likely because it was previously passaged several times in T-cell-free adherent primary Mϕ cultures. Instead, when we infected PM1 with other HIV-1_{BaL} stocks from both commercial and noncommercial sources, we observed, at various times postinfection, the spontaneous emergence of syncytiogenic, partially CD4-downregulating variants, along with the prevalent nonsyncytiogenic, non-CD4-downregulating phenotype (24). However, such variants were still unable to grow in other cell lines (24). Similar observations have been recently reported for another NSI strain (3). These results may indicate either that the virus mutates rapidly as it replicates with high efficiency in vitro or, more likely, that microvariant genotypes, already present in the initial quasispecies, gain a selective growth advantage in selected cells over time. This latter concept is corroborated by our results with a chimeric molecular clone, HXB2[BaL PvuII-BamHI], which contains a 1.3-kb fragment of the HIV-1_{BaL} envelope inserted into the HXB2 clone of HIV-1_{IIIB}. Infection of PM1 with this clone, which consists by definition of a single genotype, yielded no syncytiogenic or CD4-downregulating variants over a period of up to 4 months postinfection (24). Interestingly, the emergence of SI variants observed after prolonged growth of a typical NSI isolate in PM1 cells seems to reproduce in vitro the process of NSI→SI shifting that has been documented during the progression of HIV infection in vivo. Thus, the PM1 cell line could serve as a useful model to investigate the mechanisms responsible for this shift.

When a second chimeric virus, HXB2[BaL V3], was used, the ability of HIV-1_{IIIB} to grow in various continuous cell lines was mapped to the V3 domain of the gp120 major envelope glycoprotein. That the V3 loop could play a pivotal role in the infection process has already been suggested (10, 19, 20, 46), although there is still uncertainty regarding the exact function of this domain in the viral life cycle. The V3 loop also encompasses critical epitopes for antibody neutralization of HIV (14, 26, 31, 32). A recent report has demonstrated that Mϕ-tropic, NSI isolates possess cryptic V3 loop epitopes, at least in part inaccessible for antibody binding (3), suggesting a possible explanation for the poor susceptibility of such isolates to antibody neutralization. Similarly, we recently documented the loss of a neutralizing epitope in the V3 loop after a single spontaneous point mutation in vivo, despite efficient recognition of the linear peptide by a neutralizing MAb (43). Computer-assisted structural modeling, validated by experimental evidence, indicated that the single amino acid substitution caused a dramatic conformational change in the V3 loop, which rendered the epitope inaccessible to the MAb (43). The fact that the insertion of the HIV-1_{BaL} V3 loop into an HIV-1_{IIIB} clone was sufficient to abrogate its ability to infect continuous cell lines other than PM1 is consistent with the possibility that part of the V3 loop of HIV-1_{BaL} is inaccessible to a putative V3 ligand on the cell surface. In addition, the low number of positively charged amino acids in the V3 loop of HIV-1_{BaL} could decrease its affinity for a putative V3 ligand. It is remarkable that despite a cell line tropism similar to that of HIV-1_{BaL}, our two chimeras were still unable to efficiently replicate in primary human Mϕ. Thus, in contrast to previous results (10, 19), but in agreement with recent work from our laboratory (25), these results suggest that the V3 loop is not per se sufficient to confer true Mϕ tropism, which seems to be determined by multiple genetic elements, most likely both inside and outside the envelope.

In our experimental model, no interference was observed between Mϕ-tropic, NSI and cell-line-tropic HIV-1 isolates. This result is in contrast with previous reports which have demonstrated that receptor interference does occur between different HIV isolates (17, 41). However, other investigators demonstrated that a non-CD4-downregulating HIV-2 isolate failed to interfere with subsequent superinfection by HIV-1 (22). Interference is likely dependent on the level of residual membrane expression of the viral receptor after infection, as well as on the binding affinity of the different viral envelopes for the receptor itself. The Mϕ-tropic, NSI isolates tested in our system failed to downregulate the CD4 receptor and to induce syncytium formation in PM1 cells. Moreover, by inference from sCD4 neutralization studies reported here and elsewhere (5, 20, 28, 29, 46), virion-associated gp120 from NSI isolates appears to possess a significantly lower affinity for CD4, compared with that of cell-line-tropic, SI isolates, despite the similar CD4-binding affinities of their soluble gp120 glycoproteins. These two factors may explain why superinfection

with HIV-1_{IIIB} could occur unimpeded. This lack of interference suggests the intriguing possibility that phenotypically divergent HIV strains may directly interact in individual coinfected cells in vivo and thereby possibly generate genotypically or phenotypically mixed viral progenies. The PM1 clone may provide a helpful model for studies of virus-to-virus interaction during productive coinfection in the same target cell.

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